

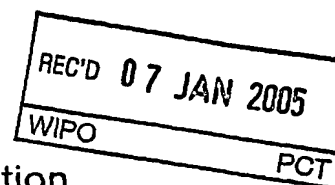
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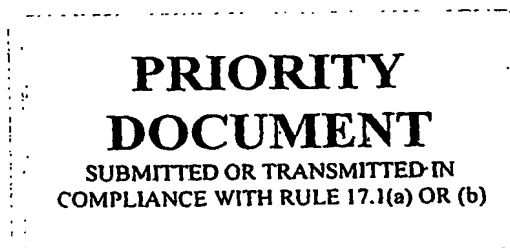
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Method for the analysis of gynecological cell proliferative disorders

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Method for the analysis of gynecological cell proliferative disorders.

The present invention relates to a method for the detection, differentiation and prognosis of a gynaecological cell proliferative disorder, comprising the following steps: a) obtaining a cervicovaginal secretion specimen from an individual b) determining the methylation status of at least one or more CpG positions c) determining from said methylation status the presence, classification and/or prognosis of a gynaecological cell proliferative disorder in said individual. The present invention further relates to a kit for performing said method.

Field of the Invention

Endometrial cancer

Endometrial cancer is one of the most common genital cancers in women worldwide. The highest incidence rates are observed in Western Europe and North America. The well known risk factors for endometrial cancer include obesity, type 2 diabetes mellitus and hypertension. Additionally, anovulation and long term use of unopposed estrogens for hormone replacement therapy increase the risk for endometrial cancer. Genetic causes of endometrial cancer are uncommon, although there is an association with hereditary non-polyposis colon cancer syndrome, in which the individual risk rises to a cumulative incidence of 40% by age 70 years. In 2001, the American Cancer Society concluded that there was insufficient evidence to recommend screening for endometrial cancer for women at average risk or increased risk due to history of unopposed estrogen therapy, nulliparity, infertility or failure to ovulate, obesity, diabetes, or hypertension. Studies examining endometrial carcinoma screening methods for asymptomatic postmenopausal women have used ultrasound-determined endometrial thickness as an indication of risk. Transvaginal ultrasonography compared with endometrial biopsy for the detection of endometrial disease had a positive predictive value of only nine percent for detecting any abnormality, with 90 percent sensitivity and 48% specificity (Langer, R. D., Pierce, J. J., O'Hanlan, K. A., Johnson, S. R., Espeland, M. A., Trabala, J. F., Barnabei, V. M., Merino, M. J., and Scully, R. E. Transvaginal ultrasonography compared with endometrial biopsy for the detection of endometrial disease. Postmenopausal Estrogen/Progestin Interventions Trial. N. Engl. J. Med., 337: 1792-1798, 1997.). There is a need for a sensitive and specific screening test for high-risk women. It has been shown that genetic abnormalities can be used to detect endometrial cancer. Changes in the status of DNA methylation are among the

most common molecular alterations in human neoplasias (Jones, P. A. DNA methylation errors and cancer. *Cancer Res.*, 56: 2463-2467, 1996.). It has been increasingly recognized over the past four to five years that the CpG islands of a large number of genes, which are unmethylated in normal tissue, are methylated to varying degrees in multiple types of human cancer (Jones, P. A. and Laird, P. W. Cancer epigenetics comes of age. *Nat. Genet.*, 21: 163-167, 1999). Aberrant methylation of CpG islands within the promoter regions of several genes such as *E-cadherin*, *adenomatous polyposis coli (APC)*, *MLH1*, *p16*, *estrogen receptor*, *progesterone receptor* and *PTEN (MMAC1)* has been identified in endometrial cancer tissue. Up to now, no investigations have been undertaken to assess the methylation status of DNA obtained from cervicovaginal secretion from endometrial cancer patients.

Long-term tamoxifen users who are at increased risk for endometrial cancer have a worse prognosis for such cancers, which seems to be due to less favorable histology and higher stage. This indicates an urgent need for a simple, non-invasive means of early detection of endometrial cancer, especially in this subgroup of women.

Cervical cancer

Cancer of the uterine cervix is an important cause of death in women worldwide. Since the introduction of PAP smears in screening programs the incidence and mortality of cervical cancer have decreased dramatically. However, successful screening strongly depends on the coverage rate of the population and the sensitivity and specificity of the screening test. A meta-analysis of studies investigating the pap test for the detection of cervical cancer and its precursors revealed a sensitivity ranging from 30% to 87% and a specificity ranging from 86% to 100%. Converging evidence from epidemiological and molecular studies suggests that infection with genital human papillomavirus (HPV³) is causally linked to the development of cervical cancer. Therefore, testing for HPV DNA has been evaluated to improve cervical cancer screening. Numerous studies showed a high sensitivity for the HPV test in detecting cervical cancer and its precursors, whereas specificity was usually lower in comparison to cytology. To reduce the inconvenience and cost of repeated clinical visits, it has been proposed that women collect cervicovaginal specimens themselves for HPV DNA assay, hopefully increasing the coverage of screening programs.

Several studies investigated HPV DNA detection rates between self-collected and physician-collected samples with varying concordance between the two different collection methods.

In addition to HPV infection, it is clear that other factors are also involved in cervical carcinogenesis because the majority of patients with HPV-associated lesions do not progress to invasive cancer. Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias. Recently, an aberrant methylation pattern was found during the multistage pathogenesis of cervical cancer with a trend to increasing methylation with increasing pathological changes (Virmani, A. K., Muller, C., Rath, A., Zochbauer-Mueller, S., Mathis, M., and Gazdar, A. F. Aberrant methylation during cervical carcinogenesis. Clin. Cancer Res., 7: 584-589, 2001).

In a study by Kinney *et. al.* Up to 60% of women diagnosed as having invasive cervical cancer were not screened despite health maintenance organization enrolment. To reduce the inconvenience and cost of repeated clinical visits it has been proposed that women collect cervicovaginal specimens themselves, thus hopefully increasing the coverage of screening programs. Previous studies investigating HPV DNA detection rates between self-collected and physician-collected samples described varying concordance between the two different collection methods. Recently, it was shown that clinician-directed swabs detect up to 28% more HPV-positive women in comparison to tampon-collected specimens. In light of the fact that numerous studies have revealed a nearly 100% sensitivity in detecting SIL and cervical cancer by HPV DNA testing of physician-collected samples, specimen collection by tampon seems not to be a feasible method for HPV DNA detection.

It has been proposed that in addition to HPV infection genetic or epigenetic alterations may be required to maintain a malignant phenotype. Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias. Recently it was suggested that aberrant methylation may play a role in cervical carcinogenesis (Virmani, A. K., Muller, C., Rath, A., Zochbauer-Mueller, S., Mathis, M., and Gazdar, A. F. Aberrant methylation during cervical carcinogenesis. Clin. Cancer Res., 7: 584-589, 2001).

Cervical cancer is the principal cause of death due to cancer in women. Five-year survival rate ranges from 15 to 80 percent, depending on the extent of the disease. Recently, several studies showed a significant reduction in the risk of relapse and death from cervical cancer, which was achieved by concurrent use of chemotherapy and radiotherapy. New predictive markers for relapse may increase survival rates by improving treatment of patients at high risk for relapse.

Several clinical and histopathological characteristics, namely tumor stage, lymph node metastasis and vascular invasion, have been shown to be prognostic factors for recurrent disease. However, new molecular and biochemical approaches for the recognition and treatment of high risk patients are needed to improve survival and avoid over-treatment of low-risk patients. The gene products of *CDH1* and *CDH13*, namely E-cadherin and H-cadherin, play a key role in cell-cell adhesion. Changes in cell-cell and cell-matrix adhesion accompany the transition from benign tumor to invasive, malignant cancer and the subsequent metastatic dissemination of tumor cells. Decrease or loss of E-cadherin expression is a common finding in many human epithelial cancers including cervical cancer. The cadherin-mediated cell adhesion system can be inactivated by several mechanisms. It has been reported that aberrant methylation of CpG islands in the E-cadherin (*CDH1*) as well as in the H-cadherin (*CDH13*) promotor or 5'-region may lead to decreased E-cadherin and H-cadherin expression. Numerous studies have demonstrated tumor-specific alterations in DNA recovered from plasma or serum of patients with various malignancies, a finding that has potential for molecular diagnosis and prognosis.

Abnormalities of cell adhesion molecule expression like E-cadherin (*CDH1*) and H-cadherin (*CDH13*) occur in various neoplastic diseases, and there is some evidence to suggest that these abnormalities are significant in the progression of certain tumor types including cervical cancer. Several mechanisms like tumor hypoxia and necrosis; stimulation of the epidermal growth factor receptor (EGFR) by EGF or TGF- α and mutations of the *CDH1* gene have been proposed for cadherin downregulation. Recently, aberrant promoter methylation of *CDH1* and *CDH13* has been described to be one of the mechanisms causing loss of or decreased E-cadherin and H-cadherin expression. Decreased E-cadherin expression has been shown to be related to enhanced metastasizing activity or more aggressive malignant tumors.

Methylated DNA has been investigated as a possible screening marker for neoplastic disease in several body fluids (Muller, H. M. and Widschwendter, M. Methylated DNA as a possible screening marker for neoplastic disease in several body fluids. *Expert Rev Mol Diagn*, 3: 443-458, 2003). However, up to now, no investigations have been undertaken to assess the methylation status of DNA obtained from cervicovaginal secretion for the assessment of patients with gynaecological cellproliferative disorders.

Description

Current methods of endometrial cancer diagnosis, namely transvaginal ultrasonography compared with endometrial biopsy for the detection of endometrial disease are estimated to have a positive predictive value of only nine percent for detecting any abnormality, with 90% sensitivity and 48% specificity. There is a need in the art for a sensitive and specific screening test for high-risk women.

The standard screening test for cervical cancer is the PAP smear. Successful screening strongly depends on the coverage rate of the population and the sensitivity and specificity of the screening test. A meta-analysis of studies investigating the pap test for the detection of cervical cancer and its precursors revealed a sensitivity ranging from 30% to 87% and a specificity ranging from 86% to 100%. However the success of screening programs aimed at detecting precancerous conditions (dysplasia) and treating them before they progress is often limited by socio-economic factors. It has been estimated that only about 5 % of women in developing countries have been screened for cervical dysplasia in the past 5 years, compared with some 40% to 50% of women in developed countries. In order to improve the successful screening of populations for cervical cancer, especially of the high-risk population with low socio-economic status, there is a need for a sensitive, specific, cost effective and self-administrable cervical cancer test.

Cervical cancer is in many cases a treatable disease, however for those patients who have poor prognosis a significant reduction in the risk of relapse and death can be achieved by concurrent use of chemotherapy and radiotherapy. However, current prognostic markers, mainly histological, only provide a limited indication of patient prognosis. Furthermore treatment of patients who are not at risk of relapse by adjuvant treatments can lead to unnecessary side effects. Therefore there exists a need in the art for improved and preferably self-administrable means of detection and prognosis of cervical cancer.

The method according to the invention provides novel sensitive and specific means for the detection, differentiation and prognosis of gynaecological cell proliferative disorders by analysis of cervicovaginal secretions. The method thereby enables specimen collection by the patient without the need for the aid of a physician. In a further aspect the invention provides methods for the analysis of said specimen for genomic methylation features associated with a

variety of gynaecological cell proliferative disorders, in particular those of the endometrium and cervix. In a further aspect the invention provides novel nucleic acids for the analysis of said cervicovaginal secretion specimen that enable the detection, differentiation and prognosis of gynaecological cell proliferative disorders. The invention further provides kits for the analysis of cervicovaginal secretions for the analysis of methylation features that enable the detection, differentiation and prognosis of gynaecological cell proliferative disorders.

The method according to the invention comprises the following steps:

- a) obtaining a cervicovaginal secretion specimen from an individual,
- b) determining the methylation status of at least one or more CpG positions,
- c) determining from said methylation status the presence, classification and/or prognosis of a gynaecological cell proliferative disorder in said individual.

The cervicovaginal secretion may be obtained by any means standard in the art, including but not limited to gynaecological swab, aspiration and cervicovaginal lavage. However in the most preferred embodiment of the method said specimen is collected by means of a tampon inserted into the individual's vaginal passage, it is preferred that the tampon is then stored in a buffer or other suitable solution. It is a particularly preferred embodiment of the invention that this step of the method is carried out by said individual, without the assistance of a physician or other healthcare professional. The solute containing DNA from the cervicovaginal may then be used in the further steps of the method.

The utility of self administered cervicovaginal specimen collection has been well established for testing of HPV (Human papilloma virus), an indicator of cervical cancer. Studies in the United States (Harper DM, Hildesheim A, Cobb JL, Greenberg M, Vaught J, Lorincz AT. Collection devices for human papillomavirus. J Fam Pract. 1999 Jul;48(7):531-5.) showed an 80% diagnosis concordance rate between specimens collected by the physician and those collected by the patient using vaginal tampons. However, the use of this technique for analysis of the diseased tissues themselves have so far, not been published.

The cervicovaginal specimen, or solution thereof is then analysed by means of a sensitive methylation assay in order to detect aberrant methylation patterns associated with the development of gynaecological cell proliferative disorders, this is enabled by analysing one or more CpG positions the aberrant methylation of which is a feature of a gynaecological cell

proliferative disorders or an indicator of prognosis thereof. In the final step of the method the presence, classification and/or prognosis of a gynaecological cell proliferative disorder is determined from said determined methylation status of one or more CpG positions.

In a preferred embodiment of the method the gynaecological cell proliferative disorder is selected from the group consisting no dysplasia or low grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, cervical cancer, endometrial cancer and grade 1 to 3 cervical intraepithelial neoplasia.

In one embodiment of the method said CpG positions are selected from one or more genes taken from the group consisting of *CDH1*, *CDH13*, *RASSF1A*, *hMLH1*, *HSPA2*, *SOCS1*, *SOCS2*, *GSTP1*, *DAPK*, *TIMP3* and *hTERT* and/or promoters, introns, first exons, regulatory elements and/or enhancers thereof. It is also a further embodiment of the invention that the sequence of said genes are selected from the group consisting of SEQ ID No: 1 to SEQ ID No: 11 according to Table 7.

In one embodiment of the method endometrial cancer is detected or differentiated from other endometrial cell proliferative disorders by analysis of one or more CpG positions of the genes *RASSF1A*, *hMLH1*, *CDH13*, *HSPA2* and *SOCS2*, in a further embodiment of said method the sequences of said genes are selected from SEQ ID Nos: 2, 3, 4, 5 and 7 according to Table 7.

In one embodiment of the method cervical cancer is detected or differentiated from other cervical cell proliferative disorders by analysis of one or more CpG positions of the genes *SOCS1*, *CDH1*, *TIMP3*, *GSTP1*, *DAPK*, *hTERT*, *CDH13*, *HSPA2*, *MLH1*, *RASSF1A* and *SOCS2*, in a further embodiment of said method the sequences of said genes are selected from SEQ ID Nos: 1 to 11 according to Table 7.

In a further embodiment of the method the prognosis of a patient with cervical cancer is determined by analysis of one or both of the genes *CDH1* and *CDH13*, in a further embodiment of said method the sequences of said genes are selected from SEQ ID Nos 1 and 2 according to Table 7. Hypermethylation of the genes are correlated with a worse prognosis of life expectancy of the patient.

Once a cervicovaginal secretion specimen is obtained from the patient according to step a) of the method genomic DNA is isolated. This may be by any means standard in the art, including the use of commercially available kits. Briefly, wherein the DNA of interest is encapsulated in by a cellular membrane the biological sample must be disrupted and lysed by enzymatic, chemical or mechanical means. The DNA solution may then be cleared of proteins and other contaminants e.g. by digestion with proteinase K. The genomic DNA is then recovered from the solution. This may be carried out by means of a variety of methods including salting out, organic extraction or binding of the DNA to a solid phase support. The choice of method will be affected by several factors including time, expense and required quantity of DNA.

The isolated genomic DNA is then analysed for aberrant methylation of CpG dinucleotides. This may be by any means standard in the art including the use of methylation sensitive restriction enzymes. However it is required that a method capable of high sensitivity be used as there is only likely to be a minimal amount of DNA from the diseased tissue present in the sample. Therefore it is particularly preferred that step b) of the method is carried out by treating the genomic DNA, or a fragment thereof, with one or more reagents to convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties. This treatment is preferably carried out by means of a bisulfite reagent (bisulfite, disulfite, hydrogen sulfite or combinations thereof) followed by alkaline hydrolysis. It is a further embodiment of the method that post-treatment the sequences to be analyzed are taken from the group consisting of SEQ ID NO: 12 to 55.

The treated genomic DNA, or the treated fragment thereof, are then preferably contacted with an amplification enzyme and at least two primers comprising, in each case a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridizes under moderately stringent or stringent conditions to a target nucleic acid. It is a further embodiment of the method that post-treatment the sequences to be analyzed are taken from the group consisting of SEQ ID NO: 12 to 55. In the final step of the method the methylation state of at least one CpG dinucleotide sequence, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences is determined based on a presence or absence of, or on a property of said amplificate whereby at least one of detecting a gynecological cell proliferative disorder, or distinguishing between gynecological cell proliferative disorders is, or providing a prognosis at least in part, afforded.

This may be carried out by any means standard in the art that enables the detection of small amounts of methylated DNA against a high background of non-methylated DNA. Particularly preferred are the MSP, HeavyMethyl (blocking oligonucleotides) and RealTime assays (including, but not limited to the Ligthcycler and MethyLight assays) and all possible combinations thereof.

The term "MSP" (Methylation-specific PCR) refers to the art-recognized methylation assay described by Herman et al. *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996 . In MSP applications, the use of methylation status specific primers for the amplification of bisulphite-treated DNA allows for distinguishing between methylated and unmethylated nucleic acids. MSP primer pairs contain at least one primer which hybridizes to a bisulphite-treated CpG dinucleotide of a pre-specified methylation state. Therefore, the sequence of said primers comprises at least one CpG, TpG or CpA dinucleotide. MSP primers specific for non-methylated DNA contain a 'T' at the 3' position of the C-position in the CpG dinucleotide. Detection of the amplificate allows for the determination of the presence of a methylated nucleic acid. The use of MSP thereby allows for the detection of a nucleic acid of a pre-specified methylation state to be amplified against a background of alternatively methylated nucleic acids (*see* figure 8 herein and the accompanying description).

In the HeavyMethyl® technique, polymerase amplification is aided by the use of blocking oligonucleotides, this technique may be used to block amplification of target nucleic acids of one methylation status thereby increasing the relative proportion of the amplificate nucleic acids of the other methylation status. The primers may be methylation specific ('MSP') or specific to non-CpG treated target nucleic acids. The methylation status of the bisulphite-treated CpG dinucleotides is determined by means of oligonucleotide blocking probes that are not displaced by the action of the polymerase, and thus block amplification of the sequence (*see* Figure 9). Non-displacement of the blocking oligonucleotides may be achieved by use of a polymerase that has no 5'-3' exonuclease activity, by use of peptide nucleic acid oligomers or by use of suitably modified oligonucleotides (e.g. a DNA oligomer lacking a free 3'-hydroxyl group)

Figure 9 shows polymerase-mediated amplification analysis of bisulfite-treated DNA ("3") corresponding to a CpG-rich genomic sequence by means of the HeavyMethyl® technique. Amplification of the treated DNA ("3") is precluded if the blocking oligonucleotide ("5")

anneals to the treated DNA as shown for the example case "B." The arrows ("1") represent primers, and dark circular marker positions ("2") on the bisulfite-treated nucleic acid strand ("3") represent methylated bisulfite-converted CpG positions, whereas white circular marker positions ("4") represent unmethylated bisulfite-converted positions. The blocking (blocker) oligonucleotides are represented by dark bars ("5"). In the example case "A," all subject genomic CpG positions were co-methylated, and both forward and reverse primers anneal to provide for unimpeded amplification of the corresponding treated nucleic acid ("3"). In the second example case "B," none of the subject genomic CpG positions were methylated, both forward and reverse primers anneal to the treated DNA sequence ("3") but are unable to amplify the sequence, because the synthesis of the complementary strand is blocked by the blocking oligonucleotide ("5") that anneals to a complementary position comprising unmethylated CpG sequences in the subject genomic DNA.

'Real time' based methods employ the use of a detection probe that hybridizes to a treated target nucleic acids during PCR amplification thereby enabling the detection of the amplified nucleic acid. The detection probes may be designed to hybridize to methylation specific target sequences by comprising one or more CpG, TpG or CpA dinucleotides. Suitable real time PCR based methods, include the art-recognized fluorescence-based real-time PCR technique MethyLight™ (Eads et al., *Cancer Res.* 59:2302-2306, 1999; U.S. Patent No. 6,331,393 to Laird et al.; and see Heid et al., *Genome Res.* 6:986-994, 1996) and the TaqMan assay. A particularly preferred embodiment comprises use of fluorescence-based Real Time Quantitative PCR (Heid et al., *Genome Res.* 6:986-994, 1996) employing a dual-labeled fluorescent oligonucleotide probe (TaqMan™ PCR, using an ABI Prism 7700 Sequence Detection System, Perkin Elmer Applied Biosystems, Foster City, California). The TaqMan™ PCR reaction employs the use of a nonextendible interrogating oligonucleotide, called a TaqMan™ probe, which is designed to hybridize to a CpG-rich sequence located between the forward and reverse amplification primers. The TaqMan™ probe further comprises a fluorescent "reporter moiety" and a "quencher moiety" covalently bound to linker moieties (e.g., phosphoramidites) attached to the nucleotides of the TaqMan™ oligonucleotide. For analysis of methylation within nucleic acids subsequent to bisulfite treatment, the probe is preferably methylation specific, as described in U.S. 6,331,393, (hereby incorporated by reference) also known as the MethyLight® assay. Variations on the TaqMan™ detection methodology that are also suitable for use with the described invention include the use of dual probe technology (Lightcycler™) or fluorescent amplification primers (Sunrise™ technology).

In the final step of the method the determination of the presence, absence, classification or prognosis is determined according to the methylation status of the analyzed CpG positions. This is carried out by reference to a pre-existing data set that defines the methylation patterns characteristic to each disease phenotype. Preferably, the correlation of the methylation status of the marker CpG positions with the phenotypic parameters is done substantially without human intervention. Machine learning algorithms automatically analyse experimental data, discover systematic structure in it, and distinguish relevant parameters from uninformative ones.

Machine learning predictors are trained on the methylation patterns at the investigated CpG sites of the samples with known phenotypical classification. The CpG positions which prove to be discriminative are used to define the reference data set. This method is successful in cancer classification (Model, F., Adorjan, P., Olek, A., and Piepenbrock, C., *Bioinformatics*. 17 Suppl 1:157-164, 2001).

Moreover, an additional aspect of the present invention is a kit comprising, for example: a bisulfite-containing reagent; a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond, are complementary, or hybridize under stringent or highly stringent conditions to a 16-base long segment of the sequences SEQ ID NOs:1 to SEQ ID NO:55; oligonucleotides and/or PNA-oligomers; as well as instructions for carrying out and evaluating the described method. In a further preferred embodiment, said kit may further comprise standard reagents for performing a CpG position-specific methylation analysis, wherein said analysis comprises one or more of the following techniques: MSP, MethyLight™ and HeavyMethyl™. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

Typical reagents (e.g., as might be found in a typical MethyLight™-based kit) for MethyLight™ analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); TaqMan® probes; optimized PCR buffers and deoxynucleotides; and Taq polymerase.

Typical reagents (e.g., as might be found in a typical MSP-based kit) for MSP analysis may include, but are not limited to: methylated and unmethylated PCR primers for specific gene

(or methylation-altered DNA sequence or CpG island), optimized PCR buffers and deoxynucleotides, and specific probes.

Typical reagents (e.g., as might be found in a typical MethyLight based kit) for HeavyMethyl analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); HeavyMethyl blocking oligonucleotides; optimized PCR buffers and deoxynucleotides; and polymerase.

Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kits (e.g., precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

The present invention shall now be further described without limitation thereof in the following examples with respect to the accompanying Figures and the attached sequence protocol. In the Figures,

Fig. 1 shows a flowchart of procedures for tampon insertion and sample preparation.

A - Tampon insertion.

B - Tampon transfer into a 50-ml tube.

C - Addition of 1.2 ml PBS buffer onto the tampon.

D - Centrifugation at 1000g for 10 min.

E - (1) shows the supernatant and (2) shows the pellet.

F - 0.2-ml aliquots of the supernatant fraction were each mixed with 0.2 ml of the working solution of the HighPure Viral Nucleic Acid kit and stored at -30°C until DNA isolation.

Fig. 2 shows the PMR values of 38 genes in patients without endometrial cancer (N=4) and with endometrial cancer (N=5) the genes *RASSF1A*, *hMLH1*, *CDH13*, *HSPA2* and *SOCS2* are highlighted with arrows.

Fig. 3 shows the methylation status of the five investigated genes (full box = methylated, empty box = unmethylated), status (endometrial cancer or no endometrial cancer) and age. * indicates cervical intraepithelial neoplasia grade III (CIN III); † indicates invasive cervical cancer.

Fig. 4 shows the ROC for detection of endometrial cancer by methylation analysis of DNA obtained from vaginal secretion for patients between 50 and 75 years of age (excluding patients with CIN III or cervical cancer, area under the curve is 0.988.

Fig. 5 shows the trend to increasing methylation from low-grade SIL to invasive cervical cancer. A statistically significant correlation between type of histology and number of methylated genes ($\rho = 0.47$, $P = 0.001$) was found. No case had 10 or 11 methylated genes. Figure 5B shows High grade SILs ($n=31$). Figure 5A shows low grade/no dysplasia SILs ($n=13$) and figure 5C shows invasive cervical cancer ($n=5$). The X axis scale shows the percentage of methylation positive samples, the Y axis shows the number of methylated genes.

Fig. 6 shows DNA methylation status of 11 genes in 49 cervicovaginal specimens was analysed using the MethyLight technique. A gene was deemed methylated if the PMR value was > 0 (Material and Methods; white and pink indicate unmethylated and methylated, respectively). Groups of patients were determined using unsupervised agglomerative hierarchical cluster analysis (average linkage, Manhattan distance) to group specimens and CpG regions. Gene names are given at the top of the figure. The inventors observed two clusters: 1 (red), 2 (blue). All patients with cervical cancer are grouped together in one cluster (blue).

Fig. 7 shows disease-free (A) and overall survival (B) according to *CDH1/CDH13* methylation status in serum samples. The lower dotted lines represent the methylated samples, the unbroken upper lines represent the unmethylated samples.

Figure 8 shows the polymerase mediated amplification of a CpG-rich sequence using methylation specific primers on four representative bisulfite-treated DNA strands (example cases "A"- "D") ("MSP Amplification"). The methylation specific forward and reverse primers ("1"), in each case, can anneal to the bisulfite-treated DNA strand ("3") if the corresponding subject genomic CpG sequences were methylated. The bisulfite-treated DNA strand ("3") can be amplified if both forward and reverse primers ("1") anneal, as shown in representative case "A" at the top of the figure.

Figure 9 shows polymerase-mediated amplification analysis of bisulfite-treated DNA ("3") corresponding to a CpG-rich genomic sequence by means of the MethylHeavy® technique.

Amplification of the treated DNA ("3") is precluded if the blocking oligonucleotide ("5") anneals to the treated DNA as shown for the example case "B."

Examples

Example 1

The following study was performed to determine whether it is possible to detect endometrial cancer by analyzing methylated DNA in cervicovaginal secretion.

Patients and Samples. A total of 124 patients were recruited for this study: 15 patients had endometrial cancer, while the no endometrial cancer group contained five patients with invasive cervical cancer, 35 with cervical intraepithelial neoplasia (CIN I, three cases; CIN II, 19 cases; CIN III, 13 cases), and 69 patients with benign disease of the uterus. Sample collection was done between 01.01.2003 and 31.05.2003 at the Department of Obstetrics and Gynecology, Innsbruck University Hospital, Austria. All patients who were scheduled to undergo surgery of the uterus on the next day including a histological diagnosis were invited to attend the study. Samples and clinical data were collected after informed consent was obtained. To ensure standardized sample collection a tampon was inserted in the patient by a physician after speculum examination and retained intravaginal for 30 minutes. Preparation of the samples is shown in Figure 1.

DNA Isolation and Methylation Analysis. Genomic DNA from samples was isolated using the *High Pure Viral Nucleic Acid Kit* (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with some modifications for multiple loading of the DNA extraction columns to gain a sufficient amount of DNA. Sodium bisulfite-treated genomic DNA was analyzed by means of MethyLight, a fluorescence-based, real-time PCR assay, as described previously. Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β -actin (*ACTB*), to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *SssI* (New England Biolabs)-treated human white blood cell DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the *GENE:ACTB* ratio of a sample by the *GENE:ACTB* ratio of *SssI*-treated white blood cell DNA and multiplying by 100. The abbreviation PMR (percentage of fully methylated reference) indicates this measurement. A gene was deemed methyl-

ated if the PMR value was > 0 . Primer and probes specific for methylated DNA and used for MethyLight reactions are listed in Table 1.

Statistical Analysis. Associations between categorical variables were tested using the Chi-Square test. Differences in median of age were examined with the Mann-Whitney U test or – between more than two groups – with the Kruskal Wallis test. Due to a significant age difference between endometrial cancer patients and the no endometrial cancer group, an age-matched group of the no endometrial cancer group with a matching ratio 1:2 was randomly selected. The computation of the Matching-Group was done using MATLAB R12 (www.mathworks.com). For determination of diagnostic accuracy a non-parametric Receiver Operating Curve (ROC) Analysis with Linear Interpolation was performed. A P-value of less than 0.05 was considered statistically significant. All statistical calculations were performed using SPSS, version 11.0, for Windows.

Results

Aberrant methylation of 38 genes in DNA obtained from vaginal secretion from the first five patients with endometrial cancer and the first four patients with benign disease was analyzed to determine appropriate genes for further study. The most appropriate genes for our further analyses were determined to be those that revealed the greatest difference in PMR values between patients with benign disease of the uterus and endometrial cancer patients (Fig. 2). Five genes, namely *RASSF1A*, *hMLH1*, *CDH13*, *HSPA2* and *SOCS2*, were selected for further analysis. DNA methylation in three or more of these five genes was observed in cervicovaginal secretion of all five patients with endometrial cancer, whereas all four patients without endometrial cancer showed no or fewer than three genes to be methylated. The inventors therefore determined the cut-off value between no endometrial cancer and endometrial cancer as methylation positive in three or more of the five investigated genes.

The overwhelming majority of the patients without endometrial cancer (99 of 109) revealed no or fewer than three genes methylated, whereas all of the 15 endometrial cancer patients had three or more genes methylated in their vaginal secretion (Fig. 3, $P < 0.001$, χ^2 test). Histological examination of the ten patients in the no endometrial cancer group with three or more genes methylated revealed invasive cervical cancer (four cases), CIN III (one case), endometrium polyp (four cases) and fibroids (one case). Samples were collected after primary surgery (curettage, punch biopsy of the cervix or hysteroscopic operation) and before second-

dary surgery (hysterectomy) in 16 out of 124 patients; 9/16 patients had endometrial cancer, 3/16 CIN III and 4/16 benign disease of the endometrium. All nine endometrial cancer patients had three or more genes methylated, the three CIN III patients revealed no methylated genes and one of the patients with benign disease showed one gene to be methylated. Within the group of patients from whom the vaginal secretion was collected prior to any surgery, one patient presented due to sonographically detected serometra with complete stenosis of the cervicouterine canal. Even this patient showed methylation of three of the five tested genes. DNA methylation of the five genes identified, seems to increase with age although statistically not significant (data not shown). Using all 15 endometrial cancer cases and 109 controls, the area under the ROC curve was 0.973 (data not shown). To rule out the possibility that abnormal DNA methylation is merely a surrogate for age rather than a cancer-specific marker, the inventors randomly age-matched two non-endometrial cancer controls for each endometrial cancer case. Investigation of DNA methylation in the cervicovaginal secretion of these 45 patients was still able to discriminate between endometrial cancer and patients without endometrial cancer ($P < 0.001$, χ^2 test) with a sensitivity of 100% and a specificity of 80%.

When analyzing all patients between 50 years and 75 years of age and excluding patients with CIN III or cervical cancer sensitivity was 100% and the specificity rose to 97.2% (Fig. 4). In this group only one out of 35 samples was false positive.

In our study all endometrial cancer patients revealed three or more of the five investigated genes methylated, whereas 99 out of 109 patients without endometrial cancer had no or fewer than three genes methylated. Four out of ten patients in the no endometrial cancer group with three or more genes methylated had invasive cervical cancer. These cases indicate that some cervical cancer patients can also be identified with this assay.

In some cases (16 out of 124) samples were collected after primary surgery and before secondary surgery. All endometrial cancer patients in this group had three or more genes methylated. These results demonstrate that aberrant methylation analysis can detect endometrial cancer even after primary surgery.

As endometrial cancer is more prevalent in older women and abnormal DNA methylation in non-malignant tissues seems to increase with age (18), the inventors especially addressed this problem within this project. Comparison of DNA methylation in the cervicovaginal secretion

of endometrial cancer patients and age-matched non-endometrial cancer controls revealed still highly significant differences between these two groups.

Endometrial cancer occurs in almost all cases after menopause. Therefore the inventors analyzed all patients between 50 years and 75 years of age and excluded patients with CIN III or invasive cervical cancer. These patients represent the group that will benefit from an endometrial cancer screening assay. In these group the sensitivity and specificity to detect patients with endometrial cancer was 100% and 97.2%, respectively.

Long-term tamoxifen users who are at increased risk for endometrial cancer have a worse prognosis for such cancers, which seems to be due to less favorable histology and higher stage. This indicates an urgent need for a simple, non-invasive means of early detection of endometrial cancer, especially in this subgroup of women.

Example 2

The aim of this study was to examine whether HPV DNA testing and methylation analysis of DNA obtained from cervicovaginal specimens, collected on a tampon, are able to detect invasive cervical cancer and whether these changes are already present in the precursor lesions.

Patients and Samples. A total of 34 patients with cervical intraepithelial neoplasia and five patients with invasive cervical cancer were included in this study. Patients were referred to our hospital for further treatment because of abnormal PAP smear or obvious cervical lesion. Patients underwent cervical conisation or in the case of obvious carcinomatous lesion cervical biopsy to obtain a histological diagnosis. Additionally, ten patients without cervical dysplasia but who underwent hysterectomy because of fibroids were investigated. All patients were taken from a prior study performed to determine whether it is possible to detect endometrial cancer by analyzing methylated DNA in cervicovaginal specimens collected by tampon (Example 1).

Sample collection Samples and clinical data were collected after informed consent was obtained. To ensure standardized sample collection a tampon was inserted in the vagina by a physician after speculum examination prior to surgery (the day before) and retained intravaginal for 30 minutes. Preparation of the samples was as described in Example 1 and Figure 1. Briefly, the tampon was transferred to a 50-ml tube after removal and 1.2 ml PBS buffer was

added to the tampon. Centrifugation at 1000g for 10min produced supernatant and a pellet. Aliquots (0.2ml) of the supernatant were each mixed with 0.2ml from the working solution of the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) and stored at -30 C until DNA isolation.

DNA isolation and methylation analysis. Genomic DNA from samples was isolated using the *High Pure Viral Nucleic Acid Kit* (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with some modifications for multiple loading of the DNA extraction columns to gain a sufficient amount of DNA. After sodium bisulfite conversion, 11 genes (*SOCS1*, *CDH1*, *TIMP3*, *GSTP1*, *DAPK*, *hTERT*, *CDH13*, *HSPA2*, *MLH1*, *RASSF1A* and *SOCS2*) underwent methylation analysis by means of the fluorescence-based, real-time PCR MethyLight assay as described previously. Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β -actin (*ACTB*), to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *SssI* (New England Biolabs)-treated human white blood cell DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the *GENE:ACTB* ratio of a sample by the *GENE:ACTB* ratio of *SssI*-treated DNA and multiplying by 100. The result is given as PMR (percentage of fully methylated reference). A gene was deemed methylated if the PMR value was > 0. Primers and probes used for MethyLight reactions are listed in Table 2.

HPV DNA analysis. HPV PCR enzyme immunoassay (PCR-EIA) was done as described by Jacobs, M. V., Snijders, P. J., van den Brule, A. J., Helmerhorst, T. J., Meijer, C. J., and Walboomers, J. M. A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J. Clin. Microbiol.*, 35: 791-795, 1997. Briefly, 10 μ l of purified total cellular DNA was employed for PCR using consensus primers GP5+/bioGP6+. The reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.5 mM MgCl₂, 200 μ M of each dNTP, 1 U of thermo-stable DNA polymerase (Taq DNA Polymerase, Roche, Vienna, Austria), and 50 pmol each of the GP5+ (5-TTTGTTACTGTGGTAGATATACTAC-3 SEQ ID NO:56) and bioGP6+ (5-GAAAAATAAACTGTAAATCATATT-3 SEQ ID NO:57) primers (MWG-Biotech, Ebersberg, Germany). A 4-min denaturation step at 94°C was followed by 40 cycles of amplification in a PCR cycler (Gene Amp PCR System 9600, Perkin Elmer, Norwalk, USA). To determine the HPV type, the PCR product was subjected to an enzyme immunoas-

say (EIA) recognizing 14 different high-risk HPV types as described by Jacobs et al.(as above).

Statistical analysis. Associations between categorical variables were tested with Pearson's chi square test (or Fisher's exact test) and the Mantel-Haenzel Test. Correlations between ordinal variables were evaluated using the Spearman Rank Correlation Coefficient. For unsupervised hierarchical clustering of clinical cases and genes the inventors used the complete linkage aggregation method and the Manhattan distance function.

All statistical calculations were performed using SPSS, version 11.0, for Windows and Gene Expression Similarity Suite (<http://genome.tugraz.at/Software/Genesis/Genesis.html>).

RESULTS

This study investigated cervicovaginal specimens sampled by intravaginal tampon application in patients without cervical dysplasia, patients with low-grade and high-grade SIL and patients with invasive cervical cancer for HPV DNA and aberrant methylation of 11 genes. High-risk HPV DNA was detected in 2/3, 21/31, and 3/5 in low-grade SIL, high-grade SIL and invasive cervical cancer, respectively (Table 3). Patients without cervical dysplasia showed no HPV infection. The three HPV-positive cervical cancers were large cell squamous cancers, whereas both HPV-negative cases were small cell cervical cancers. Differences in methylation of the investigated genes between the non-dysplasia group and the low-grade SIL group were not statistically significant. Therefore, the results from these two groups were combined and compared with the high-grade SIL and invasive cancer groups. An overview of the frequency of methylated genes is given in Table 3. All investigated genes except *GSTP1* and *SOCS2* were significantly more frequently methylated in high-grade SIL and/or invasive cancer in comparison to the non-dysplasia/low-grade SIL group. *CDH1* and *SOCS2* were found to be methylated in nearly a quarter of the non-dysplasia/low-grade SIL patients whereas *hTERT* was methylated exclusively in specimens obtained from cervical cancer patients. No methylated genes were found in 61% and 42% of the cervicovaginal specimens from patients with non-dysplasia/low-grade SIL and high-grade SIL, respectively, whereas cervical cancer patients revealed five or more methylated genes in each investigated sample. The percentage of methylation-positive samples showed a significantly increasing trend from non-dysplasia/low-grade SIL to invasive cervical cancer, as shown in Fig. 5. No significant correlation between HPV positivity and aberrant hypermethylation was observed ($p = 0.295$,

Fisher's exact test). HPV DNA-positive samples and/or at least one methylated gene were found in 46% (7/13; non-dysplasia/low-grade SIL), 94% (29/31; high-grade SIL), and 100% (5/5, invasive cancer) of samples (Table 3).

Two clusters were formed by unsupervised hierarchical cluster analysis using solely information on the DNA methylation of the 11 genes tested. One of the two clusters contained all five invasive cancers as well as two high-grade SILs (Fig. 3).

hTERT was never found to be methylated in cervical intraepithelial neoplasias, whereas 80% of the specimens obtained from cervical cancer patients were methylated. This finding suggests that methylation of *hTERT* is a late event in cervical carcinogenesis. *SOCS2* and *CDH1* were methylated in nearly a quarter of patients from the non-dysplasia/low-grade SIL groups, indicating that methylation of these genes is an early event in cervical carcinogenesis. Our results clearly show that an increasing percentage of methylation-positive samples is associated with increasing pathological changes of the cervix uteri (Fig. 5), suggesting that methylation, in addition to HPV infection, is an important factor in cervical carcinogenesis. HPV DNA-positive samples and/or at least one methylated gene were found in more than 90% of samples from patients with high-grade SIL and in 100% of patients with invasive cervical cancer. No significant correlation between HPV positivity and aberrant hypermethylation was observed. It can be speculated that aberrant methylation in women with or without HPV infection may help identify subgroups at increased risk for histological progression or cancer development. Our study shows that it is possible to detect HPV and aberrant hypermethylation of various genes in DNA from tampon-collected samples. The inventors were able to identify all invasive cervical cancers. Both methods combined detect a high percentage of high-grade cervical lesions.

Example 3

In the following study the inventors investigated the methylation status of *CDH1* and *CDH13* in serum samples of cervical cancer patients for their utility as prognostic markers.

Patients and Samples

A total of 93 patients with invasive cervical cancer (age 26-96 years, median 52 years), all treated at the Department of Obstetrics and Gynecology, Innsbruck University Hospital, between 1990 and 1998 were included in this study. Serum samples were taken on the date of

diagnosis and before initial treatment. These serum samples were taken from a prior study investigating the presence of serum human papillomavirus DNA in cervical cancer patients.

Major clinical and histopathological characteristics of patients are given in Table 4. Treatment was according to international standards. None of the patients received concurrent chemotherapy and radiotherapy. All patients were followed up after primary treatment at our department, namely at intervals increasing from three months to one year until death or end of the study. The follow-up period ranged from one month to 12.4 years (median 3.5 years).

DNA isolation and methylation analysis

Serum samples (300µl) were treated with SDS and proteinase K (300µl of 1% SDS, 500µg/ml proteinase K) at 55°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA. The DNA was re-suspended in 80µl LoTE buffer (30mM Tris and 0.3mM EDTA). Sodium bisulfite conversion of genomic DNA was performed as described previously.¹² Sodium bisulfite-treated genomic DNA was analysed by means of the MethyLight, a fluorescence-based, real-time PCR assay, as described previously. Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β -actin (*ACTB*), to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *SssI* (New England Biolabs)-treated human white blood cell DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the *GENE:ACTB* ratio of a sample by the *GENE:ACTB* ratio of *SssI*-treated white blood cell DNA and multiplying by 100. The abbreviation PMR (percentage of fully methylated reference) indicates this measurement. For each MethyLight reaction 10µl of bisulfite-treated genomic DNA were used. A gene was deemed methylated if the PMR value was > 0. To verify the reproducibility of each assay the normalized value (*Gene: ACTB*) of the standard sample was compared between the different PCR runs. The following primers and probes were used for MethyLight reactions: *CDH1*: 5'-AATTTTAGGTTAGAGGGTTATCGCGT-3' SEQ ID NO: 58 (forward primer), 5'-TCCCCAAAACGAACTAACGAC-3' SEQ ID NO: 59 (reverse primer), 5'-FAM-CGCCCACCCGACCTCGCAT-BHQ-1-3' SEQ ID NO: 60 (probe); *CDH13*: 5'-AATTCGTTCGTTTTGTGCGT-3' SEQ ID NO: 61 (forward primer), 5'-CTACCCGTACCGAACGATCC-3' SEQ ID NO: 62 (reverse primer), 5'-FAM-AACGCAAAACGCGCCCGACA-BHQ-1-3' SEQ ID NO: 63 (probe).

Statistical analysis

Associations between categorical variables were tested with Pearson's chi square test. The Kaplan-Meier method was used for univariate survival analysis, and the log rank test was used to assess the difference between survival curves. Cox's proportional hazards analysis was used to estimate the prognostic effects of various variables. A P value of less than 0.05 was considered statistically significant. These statistical calculations were performed using SPSS, version 11.0, for Windows.

RESULTS

Aberrant promoter hypermethylation of *CDH1* and *CDH13* was observed in 42% (39 out of 93) and 4% (4 out of 93), respectively (Table 4). Three of the *CDH13* methylation positive serum samples also revealed *CDH1* methylation. The inventors therefore collapsed *CDH1* and/or *CDH13* methylation for further analysis. *CDH1* methylation was predominantly observed in FIGO stage III, whereas distribution of *CDH1* and *CDH13* methylation within the other clinical and histopathological parameters showed no significant differences (Table 4).

To determine whether any prognostic significance was attached to differences in *CDH1* and/or *CDH13* methylation, the inventors compared the clinical outcome of cervical cancer patients with *CDH1/CDH13* methylation status. A trend to poorer overall survival for patients with methylated *CDH1/CDH13* was observed ($P = 0.09$) (Figure 7B). Cervical cancer patients with unmethylated *CDH1/CDH13* in serum samples taken before treatment revealed a statistically significant better disease-free survival in comparison to patients with methylated *CDH1/CDH13* ($P = 0.03$) (Figure 7A). Median disease-free survival for *CDH1/CDH13* methylation negative and positive patients was 4.3 years and 1.2 years, respectively.

To assess independent prognostic significance a Cox proportional hazard model analysis was performed including tumor stage, histology, grade of differentiation, age and *CDH1/CDH13* methylation status. In addition to tumor stage and age only *CDH1/CDH13* methylation status ($P = 0.005$) turned out to be of independent prognostic significance for disease-free and overall survival in cervical cancer patients (Tables 5 and 6). Serum *CDH1/CDH13* methylation positive patients had a more than twofold risk for relapse and death than did *CDH1/CDH13* methylation negative patients.

Distribution of *CDH1* and *CDH13* methylation within the clinical and histopathological parameters showed no significant differences except *CDH1* methylation was predominantly observed in advanced FIGO stage. Therefore, no difference in *CDH1* methylation according to FIGO stage could be observed. The higher methylation frequency of *CDH1* with increasing tumor stage and the association of *CDH1* and *CDH13* methylation in serum samples with enhanced relapse frequency in our study confirms these results. Up to now it has not been investigated whether one of the above mentioned mechanisms alone or a combination of these mechanisms causes loss of cadherin expression. DNA recovered from plasma or serum of patients with various malignancies reflects tumor-specific genetic and epigenetic alterations like methylation of the primary tumor (Ziegler A, Zangemeister-Wittke U, Stahel RA. Circulating DNA: a new diagnostic gold mine? Cancer Treat Rev 2002;28:255-71.). The simple procedure of blood drawing in combination with a high-throughput analysis like MethyLight opens a feasible approach for a possible routine use of these markers. Inactivation of the cadherin-mediated cell adhesion system, caused by aberrant methylation, is a common finding in human cancers. Therefore, investigation of *CDH1* and *CDH13* methylation as a prognostic parameter in serum samples from patients with various malignancies could be of interest. Our study revealed that *CDH1/CDH13* methylation is an independent prognostic parameter for both disease free and overall survival in cervical cancer patients.

Table 1: Primers and probes used according to Example 1

HUGO Gene Nomenclature	Forward Primer Sequence	Reverse Primer Sequence	Probe Oligo Sequence
ACTB	TGTTGATGAGGAGGTTAGTAAGT	AACCAATAAAACCTACTCTCTCTTAA	6FAM-ACCAACACCAACCAACCAACCAACCA-BHQ-1
APC	GAACCAAAACGCTCCCAT	TTATATGTGCTGTTACGTGGTTTATAT	6FAM-CCCGTGCAGAAACCGCGGATT-BHQ-1
ARH1	GCGTAAGCGGAATTTATGTTGT	CCGGAGATTTATATTCGAGCTT	6FAM-CGCACACAAACGAAATACGAAACGCA-BHQ-1
BLT1	GCGTTGTTTATCGGAAG	AAACGTAATTCGCGCTCG	6FAM-GACTCCGCGCAACTTCGCCAAA-BHQ-1
BRCA1	GAGAGGTTGTTTATAGCGGTAGT	CGCGCAATCGCAATTTTAAT	6FAM-CCGCGCTTTTCGTTACCAACGA-BHQ-1
CALCA	GTTTGGAGTATGAGGGTGAGC	TTCCGCGCGCTATAAATCG	6FAM-ATTCCGCAATACACAAACCAATAAACG-BHQ-1
CDH1	AATTTAGGTTAGAGGGTTATCGGT	TCCCAAAACGAACTAACGAC	6FAM-CCGCAACCGCACTCGCAT-BHQ-1
CDH13	AATTTGTTGTTTGTGGT	CTACCGTACCGAAGATCC	6FAM-AAGCGCAAAACGCGCGGCA-BHQ-1
CDKN2A	TGGAGTTTGGAGGGAGTT	AACAACGCGCGCACTCTCT	6FAM-ACCGCAACCGCAACCGCG-BHQ-1
CYP11B1	GTGCGTTTGGAGGGAGTT	AACGCAACGCGCACTCTCT	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
DAPIK1	TGTCGTGCTTTCGTTAGTT	AACGCAACGCGCACTCTCT	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
ESR1	GGCGTTGTTTGGGATG	TCGCGCTAGAAACGTTT	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
ESR2	TTTGAATTTGAGGGCGAAGTAG	AACGTCGCTATTAGTCGCTT	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
FGF18	ATCTCTCTCTCTCTCTCT	AACGTCGCTATTAGTCGCTT	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
GSTM3	GCG3 CAA CCG CCG TAA CT	AACTACGACGAGCAACTCCAA	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
GSTM1	GTGCGGTGCTGATTAGTATG	CCGACGCTCTCTCTCTCT	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
HIC1	GTAGCGGTTAGGGGCTC	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
HLA-G	CAC CCG CAT ATA CCG GCT AA	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
HSD17B4	TATGTTGAGGTTGCAAGG	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
HSPA2	CAC GAA CAC TAC CAA CAA CTC AAC T	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
IGFBP2	CTC GCG CCG ACA AAT AAA TAC	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
MGMT	GCGTTTGGAGGTTGAGT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
MILH1	AGGAAGGCGGATAGGATT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
MILL7	CCT CAC GAT ACC TCC CCT CAA	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
MT3	CGA TAA ACG AAC TTC TCC AAA CAA	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
MYOD1	GAGCGCGGTAGTAGCG	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
PGR	TTATAATCGAGGCGGTAGTGT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
PP1R13B	CCT CAC CCA CCG ACA TCA TC	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
PTGS2	CGGAAGCGTTGGGTAAG	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
RASSF1A	ATTGAGTTGCGGAGTTGGT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
SOC1	GCGTCAGTTGCGGTATTT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
SOC2	TCC CTT CCG CCG CAT T	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
SYK	GGGCGGATATTGGAG	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
TER1	GGATTCGCGGTATAGCGTT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
TFP1	TAAGGTTACGTTGGTTATTTGTTGA	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
TIMP3	GCGTCGAGGTTAGGTTGTT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
TITF1	CGA AAT AAA CCG AAT CCT CCT TAA	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
TP53BP2	ACC CCG TAA CCG GAC TTT ATC	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1
TWIST	GTAGCGCGCGCAAGCT	GGG AGC GGA TTG GGT TTG	6FAM-CCGCGCAACCGCAACCGCG-BHQ-1

Table 2: Primers and probes used according to Example 2

Gene	Forward Primer	Reverse Primer	Probe
<i>SOC</i>	GCGTCGAGTTCGTGGG	CCGAAACCATCTTCA	6FAM-
<i>S1</i>	TATTT	CGCTAA	ACAATTCCGCTAACGACTATCGCC
<i>CDH</i>	AATTTTAGGTTAGAGG	TCCCCAAAACGAAAC	6FAM-CGCCCCACCCGACCTCGCAT
<i>1</i>	GTTATCGCGT	TAACGAC	BHQ-1
<i>TIMP</i>	GCGTCGGAGGTTAAGG	CTCTCCAAAATTACC	6FAM-AACTCGCTCGCCCGCCGAA
<i>3</i>	TTGTT	GTACGCG	BHQ-1
<i>GST</i>	GTCGGCGTCGTGATTT	AAACTACGACGACG	6FAM-
<i>P1</i>	AGTATTG	AAACTCCAA	AAACCTCGCGACCTCCGAACCTTA
<i>DAP</i>	TCGTCGTCGTTTCGGTT	TCCCTCCGAAACGCT	6FAM-
<i>K1</i>	AGTT	ATCG	CGACCATAAACGCCAACGCCG-
<i>hTER</i>	GGATTCGCGGGTATAG	CGAAATCCGCGCGAA	6FAM-
<i>T</i>	ACGTT	A	CCCAATCCCTCCGCCACGTAAAA-
<i>CDH</i>	AATTCGTTCGTTTTGT	CTACCCGTACCGAAC	6FAM-
<i>13</i>	GCGT	GATCC	AACGCAAAACGCGCCCGACA-
<i>HSP</i>	CACGAACACTACCAAC	GGGAGCGGATTGGGT	6FAM-
<i>A2</i>	AACTCAACT	TTG	CCGCGCCCAATTCCCGATTCT-
<i>MLH</i>	AGGAAGAGCGGATAG	TCTTCGTCCCTCCCTA	6FAM-
<i>1</i>	CGATTT	AAACG	CCCGCTACCTAAAAAAATATACG
<i>RASS</i>	ATTGAGTTGCGGGAGT	ACACGCTCCAACCGA	6FAM-CCCTTCCCAACGCGCCCA-
<i>F1A</i>	TGGT	ATACG	BHQ-1
<i>SOC</i>	TCCCTTCCCCGCCAT T	TTGTTTTTGTGCGGGT	6FAM-
<i>S2</i>		GATTT	CCGAAAAACTCAAAACACCGCA
<i>ACT</i>	TGGTGATGGAGGAGGT	AACCAATAAAACCTA	6FAM-
<i>B</i>	TTAGTAAGT	CTCCTCCCTTAA	ACCACCACCAACACACAATAAC
			AAACACA-BHQ-1

Table 2 Methylated genes and high-risk HPV DNA in patients with no dysplasia/low-grade SIL, high-grade SIL and invasive cervical cancer

Variables	No dysplasia/low-grade SIL n=13	High-grade SIL n=31	Invasive Cancer n=5	p
Genes				
<i>SOCS1</i> methylated	0%	7%	60%	< 0.001 (*)
<i>CDH1</i> methylated	23%	39%	100%	0.011 (*)
<i>TIMP3</i> methylated	0%	16%	100%	< 0.001
<i>GSTP1</i> methylated	0%	7%	20%	0.282
<i>DAPK</i> methylated	8%	23%	80%	0.006 (*)
<i>hTERT</i> methylated	0%	0%	80%	< 0.001 (*)
<i>CDH13</i> methylated	8%	13%	100%	< 0.001 (*)
<i>HSPA2</i> methylated	0%	3%	60%	< 0.001 (*)
<i>MLH1</i> methylated	0%	3%	40%	0.004
<i>RASSF1A</i> methylated	8%	0%	40%	0.002
<i>SOCS2</i> methylated	23%	45%	60%	0.26
At least one gene methylated	39%	58%	100%	0.06
High-risk HPV DNA positive	15%	68%	60%	0.006
At least one gene methylated and/or high-risk HPV DNA-positive	46%	94%	100%	0.001

TABLE 4 - Methylation of *CDH1* and *CDH13* in serum samples of cervical cancer patients

Characteristics	n ^a	CDH1	CDH13
Stage			
FIGO I	23	30%	0%
FIGO II	24	29%	4%
FIGO III	33	67% ^b	6%
FIGO IV	13	23%	8%
Tumor grade			
1	22	50%	0%
2	50	34%	6%
3	16	56%	6%
Histology			
squamous	84	42%	5%
adeno/adenosquamous	9	44%	0%
Age			
< 50	38	36%	5%
≥ 50	55	46%	4%
Total	93	42%	4%

Tumor grade was unknown in five cases

^a n, number of cases examined.

^b P = 0.005 (chi² test)

TABLE 5 - Multivariate analysis for risk of relapse

Variable	Relative risk of relapse (95% CI)	P value
Stage		<0.001
FIGO II (vs. FIGO I)	0.6 (0.2-1.6)	0.316
FIGO III (vs. FIGO I)	1.3 (0.6-2.9)	0.524
FIGO IV (vs. FIGO I)	8.6 (3.3-22.3)	<0.001
Tumor grade		0.132
grade 2 (vs. grade 1)	1.9 (0.9-4.2)	0.092
grade 3 (vs. grade 1)	2.1 (0.9-4.8)	0.065
Histology		
squamous (vs. adeno/adenosquamous)	0.6 (0.2-1.7)	0.303
Age	0.97 (0.95-0.99)	0.012
<i>CDH1</i> and/or <i>CDH13</i> methylated (vs. unmethylated)	2.5 (1.3-4.6)	0.005

TABLE 6 - Multivariate analysis for overall survival

Variable	Relative risk of death (95% CI)	P value
Stage		<0.001
FIGO II (vs. FIGO I)	0.7 (0.3-1.8)	0.504
FIGO III (vs. FIGO I)	1.0 (0.4-2.5)	0.993
FIGO IV (vs. FIGO I)	11.1 (4.0-30.4)	<0.001
Tumor grade		0.07
grade 2 (vs. grade 1)	2.3 (1.1-5.0)	0.037
grade 3 (vs. grade 1)	2.5 (1.0-6.1)	0.041
Histology		
squamous (vs. adeno/adenosquamous)	1.3 (0.4-4.3)	0.645
Age	1.0 (0.9-1.0)	0.146
<i>CDH1</i> and/or <i>CDH13</i> methylated (vs. unmethylated)	2.5 (1.3-4.8)	0.005

Table 7: Genes and equivalent sequences according to the invention.

Gene/Associated gene	Genomic sequence SEQ ID NO:	Converted sequence SEQ ID NO:
CDH1	1	12, 13, 34 and 35
CDH13	2	14, 15, 36 and 37
RASSF1A	3	16, 17, 38 and 39
hMLH1	4	18, 19, 40 and 41
HSPA2	5	20, 21, 42 and 43
SOCS1	6	22, 23, 44 and 45
SOCS2	7	24, 25, 46 and 47
GSTP1	8	26, 27, 48 and 49
DAPK1	9	28, 29, 50 and 51
TIMP3	10	30, 31, 52 and 53
hTERT	11	32, 33, 54 and 55

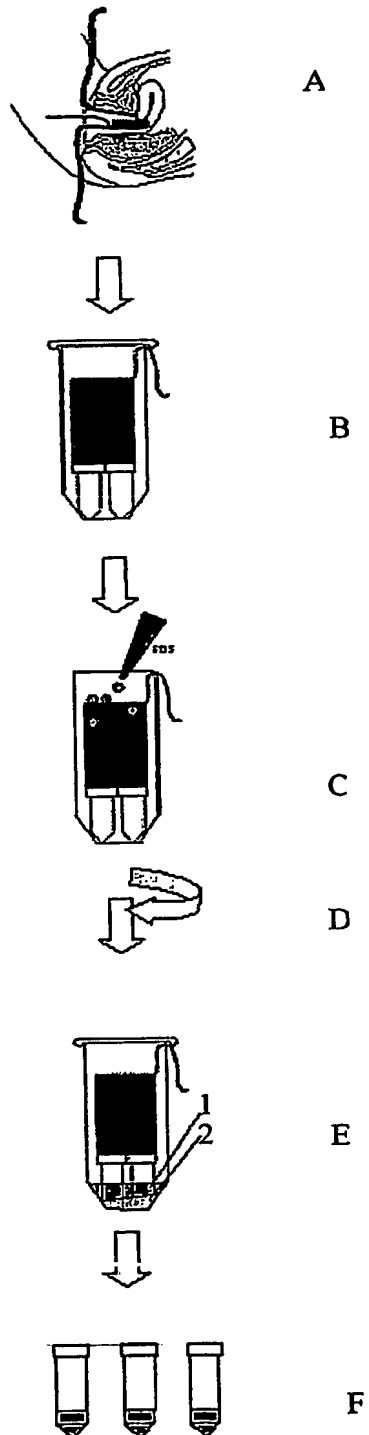
Claims

1. A method for the detection, differentiation and prognosis of a gynaecological cell proliferative disorder comprising the following steps:
 - a) obtaining a cervicovaginal secretion specimen from an individual
 - b) determining the methylation status of at least one or more CpG positions
 - c) determining from said methylation status the presence, classification and/or prognosis of a gynaecological cell proliferative disorder in said individual.
2. The method according to claim 1 wherein said CpG positions are selected from one or more genes taken from the group consisting of CDH1, CDH13, RASSF1A, hMLH1, HSPA2, SOCS1, SOCS2, GSTP1, DAPK, TIMP3 and hTERT.
3. The method according to claim 1 wherein said CpG positions are selected from one or more genes taken from the group consisting of SEQ ID No: 1 to SEQ ID No: 11.
4. A method according to claim 1 wherein in step a) the specimen is obtained by one or more of the following methods gynaecological swab, aspiration, cervicovaginal lavage and tampon based collection.
5. The method according to claims 1 and 2 wherein the gynaecological cell proliferative disorder is selected from the group consisting no dysplasia or low grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, cervical cancer, endometrial cancer and grade 1 to 3 cervical intraepithelial neoplasia.
6. The method according to claim 1 wherein hypermethylation of said genes correlates with bad prognosis.
7. The method according to claim 1 wherein step b) comprises the following steps
 - a) treating the genomic DNA, or a fragment thereof, with one or more reagents to convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties;

- b) contacting the treated genomic DNA, or the treated fragment thereof, with an amplification enzyme and at least two primers comprising, in each case a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridizes under moderately stringent or stringent conditions to a target nucleic acid, and
 - c) determining, based on a presence or absence of, or on a property of said amplificate, the methylation state of at least one CpG dinucleotide sequence, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences, whereby at least one of detecting a gynecological cell proliferative disorder, or distinguishing between gynecological cell proliferative disorders is, or providing a prognosis at least in part, afforded.
8. The method according to claim 7 wherein in step ii) the target nucleic acid is selected from SEQ ID No: 12 to SEQ ID No: 33.
9. A kit useful for detecting, or for detecting distinguishing between or among gynecological cell proliferative disorders of a subject, comprising:
at least one of a bisulfite reagent, or a methylation-sensitive restriction enzyme; and
at least one nucleic acid molecule or peptide nucleic acid molecule comprising, in each case a contiguous sequence at least 9 nucleotides that is complementary to, or hybridizes under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID 12 to SEQ ID NO 55; and complements thereof.
10. The kit of claim 9, further comprising standard reagents for performing a methylation assay selected from the group consisting of MSP, MethyLight and HeavyMethyl and combinations thereof.

Abstract

The present invention relates to a method for the detection, differentiation and prognosis of a gynaecological cell proliferative disorder, comprising the following steps: a) obtaining a cervicovaginal secretion specimen from an individual b) determining the methylation status of at least one or more CpG positions c) determining from said methylation status the presence, classification and/or prognosis of a gynaecological cell proliferative disorder in said individual. The present invention further relates to a kit for performing said method.

Figure 1

2/19

Figure 2

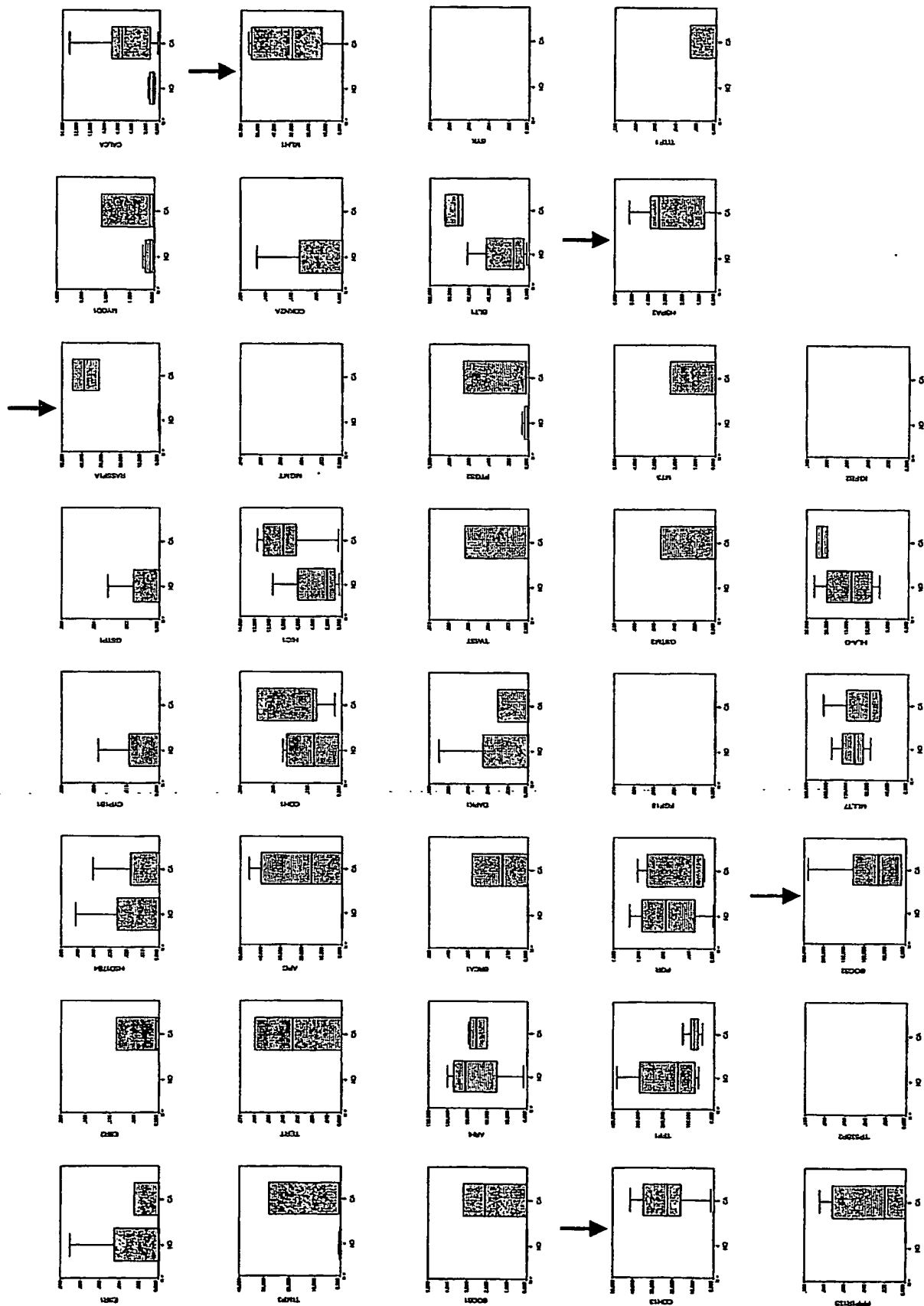


Figure 3

Genes						Endometrial cancer/ no		Age (years)
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						endometrial cancer	52	
						endometrial cancer	56	
						endometrial cancer	60	
						endometrial cancer	62	
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						endometrial cancer	63	
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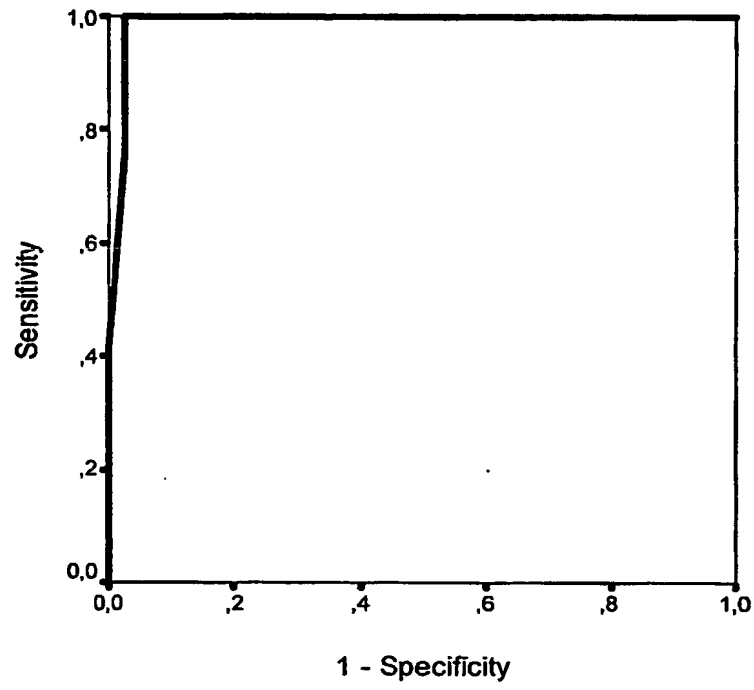
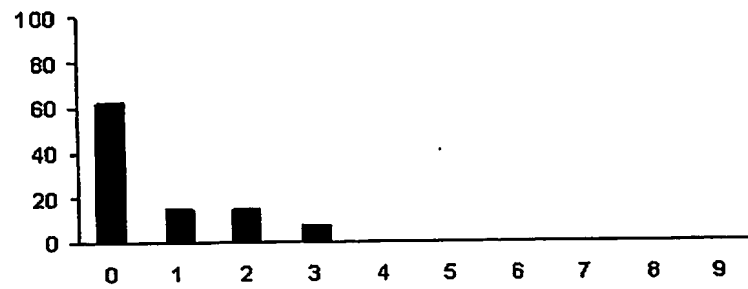
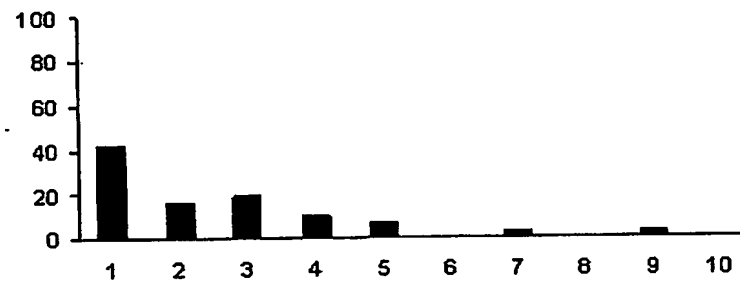
Figure 4

Figure 5

A



B



C

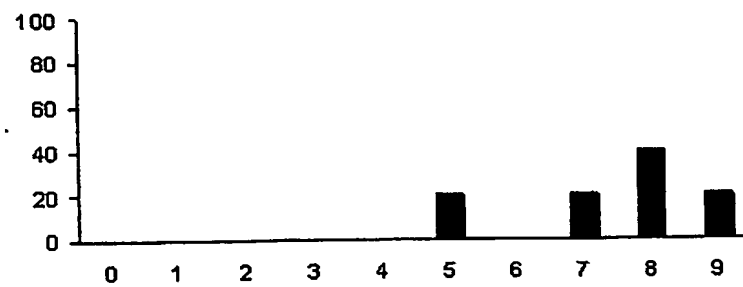


Figure 6

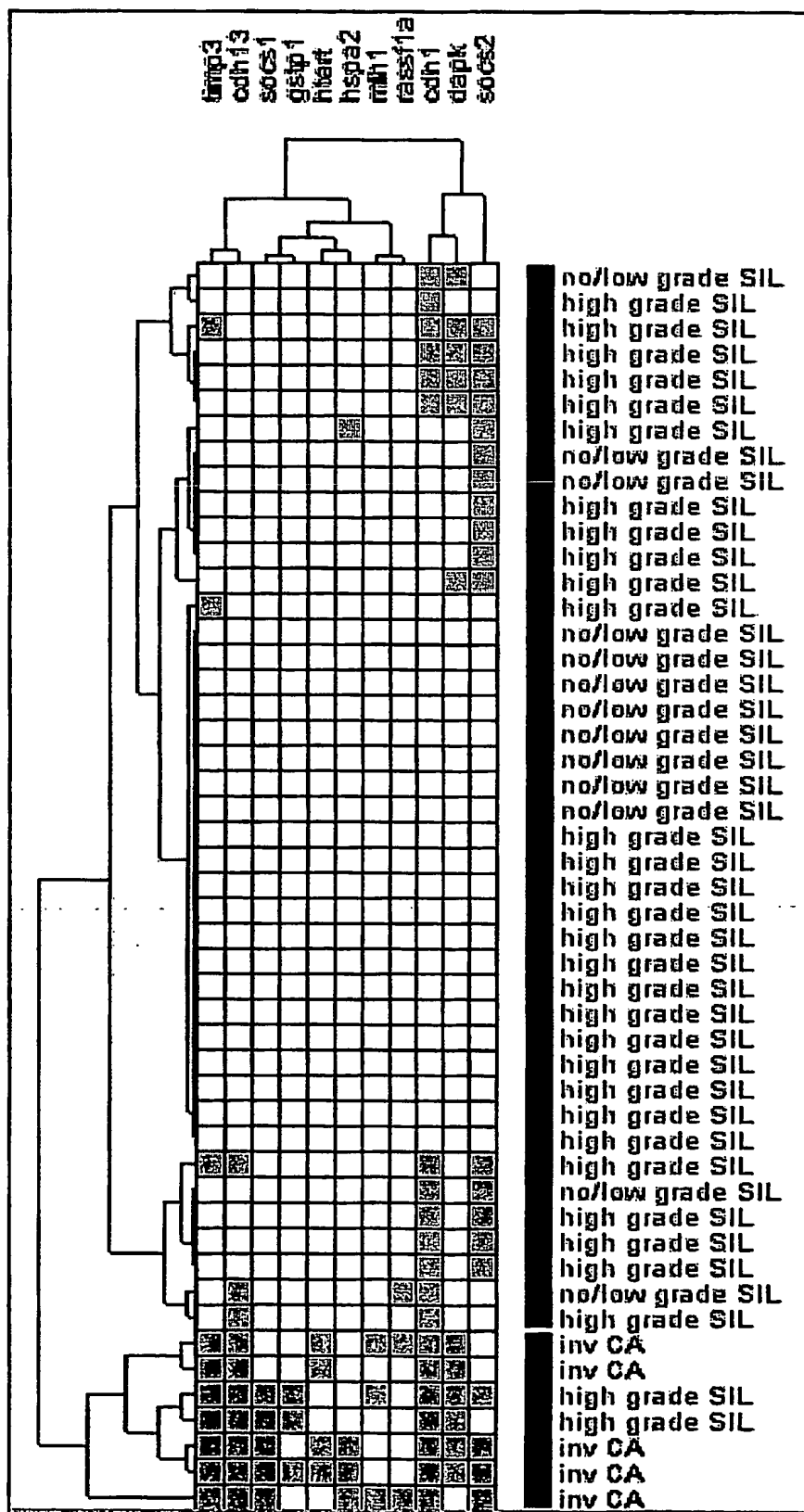
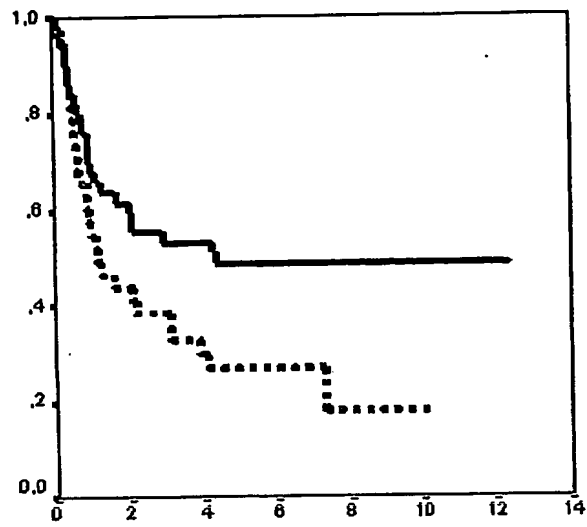


Figure 7

A



B

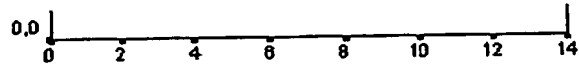


Figure 8

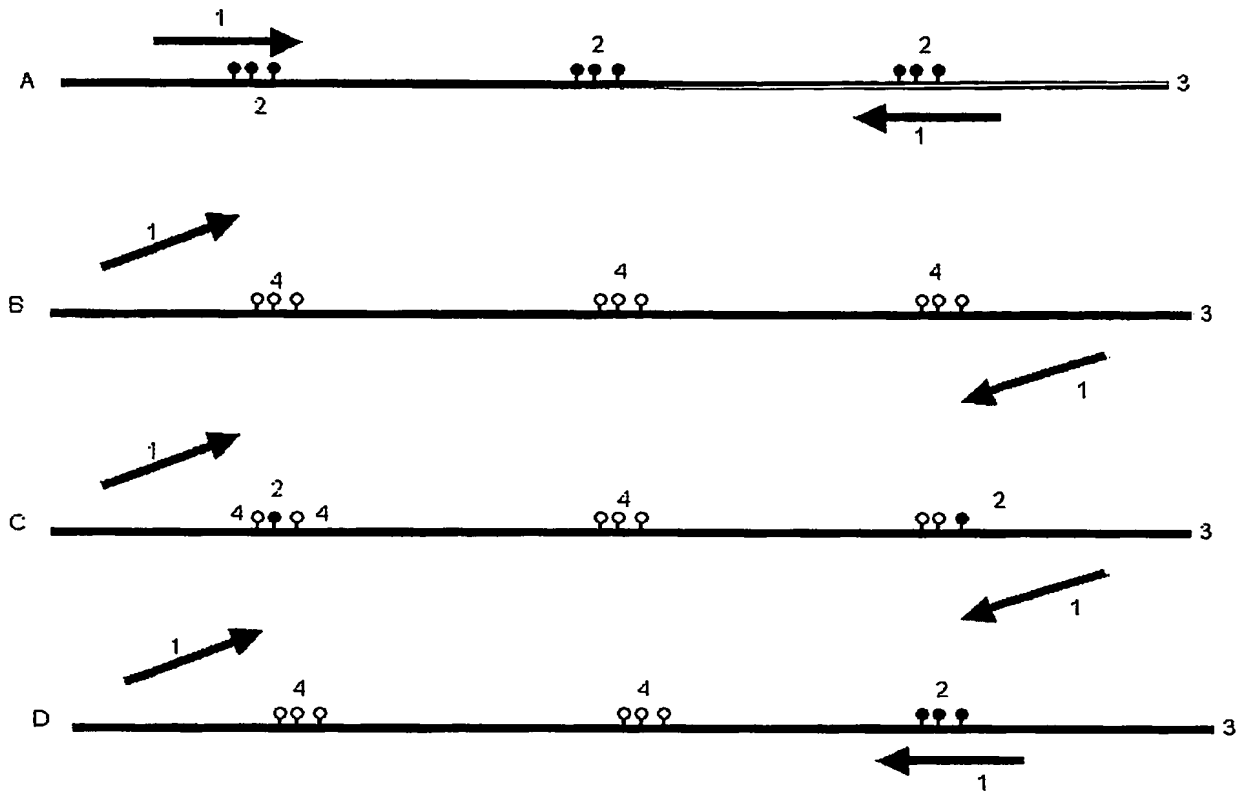
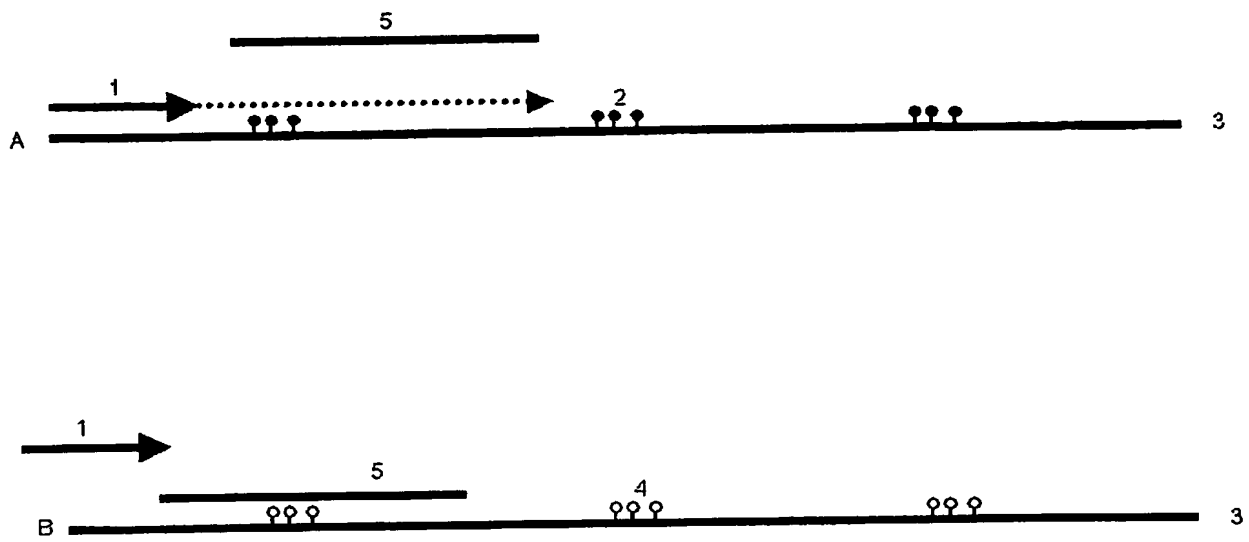


Figure 9



Sequence listing

EPO - Munich
41
10. Nov. 2003

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<120> Method for the analysis of gynaecological cell proliferative disorders.

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<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<211> 3190

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<223> chemically treated genomic DNA (Homo sapiens)

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<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 16

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<210> 17
<211> 2501
<212> DNA
<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 17

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<210> 18

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 18

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<210> 19

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 19

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<211> 3838

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<211> 3838

<212> DNA

<213> Artificial Sequence

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<223> chemically treated genomic DNA (Homo sapiens)

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<211> 2893

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<211> 2893

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 26

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 26

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<210> 27
 <211> 2501
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 27

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<210> 28
 <211> 2501
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 28

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<210> 29

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 29

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<211> 4501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<211> 3190

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 34

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<210> 35

<211> 3190

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 35

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<210> 36

<211> 2301

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 37

<211> 2301

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 37

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<210> 38

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 38

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<210> 39

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 39

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<210> 40

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 40

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<210> 41

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 41

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<211> 3838

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 43

<211> 3838

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 43

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<210> 44

<211> 2893

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 44

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<210> 45

<211> 2893

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<211> 5898

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 46

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<211> 5898

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 48

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 48

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<210> 50

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 50

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<210> 51

<211> 2501

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 51

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<210> 52
 <211> 4501
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 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 52

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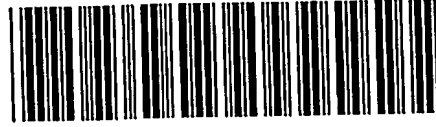
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